

USE OF ADENOVIRUSES MUTATED IN THE VA GENES FOR CANCER TREATMENT

The field of the invention relates, in general terms, to the tumor biology field. Particularly, the invention is related to adenoviruses mutated VA RNAs genes and their use for inhibiting cancer.

STATE OF PRIOR TECHNIQUE

The current cancer treatment is mainly based on chemotherapy, radiotherapy and surgery. Although cancer has an elevated rate of recovery for the early stages, most advanced cancer cases are incurable because they cannot be removed by surgery or because the administered radio or chemotherapy doses are limited by their toxicity in normal cells. Genetic material transference for inhibiting or destroying tumors presents a very promising therapeutic alternative. Compared to conventional strategies, this gene therapy strategy seeks to be more specific for malignant cells attacking the genetic defects of tumor cells. There are some strategies that use DNA as a therapeutic agent: the transfer of genes that stimulate anti-tumor immune responses, the transfer of toxic genes that activate drug toxicity, DNA transfer for blocking or reestablishing the expression of the genes involved in tumors development (oncogenes, tumor suppressor genes, antiangiogenic genes, etc). In addition to therapeutic DNA, the other element of the gene therapy is the vehicle that transports this DNA: the vector. Synthetic vectors and virus derivatives have been used to increase the DNA transfer to target cells. The latter are generally more efficient for transferring DNA or transducing tumor cells. Viral vectors have been developed from different types of viruses from retroviruses, the Simple Herpes virus, adeno-associated virus and adenovirus among others. In cancer gene therapy, the adenovirus has been preferred because of its high capacity of infecting epithelial cells that are supposed to be the origin of most of the solid tumors. Other advantages of adenoviral vectors are that they can transfer DNA to cells that are not in division, that the DNA of the vector is not integrated into the genome of the transduced cell, that these vectors may be



purified to concentrations of 10^{13} viral particles per milliliter and that they are stable in the blood stream since they lack a lipid coating.

Adenovirus is a DNA virus without a lipid coating that is characterized by having an icosaedric capsid packing a double-strand linear DNA of approximately 36 kilobases. There are 50 serotypes of human adenoviruses classified in six subgroups (A to F) according to their structural and functional properties, such as the agglutination of erythrocytes. In gene therapy, the 5 type adenovirus has been preferred because it is well characterized molecularly and because of its low pathogenicity in humans. In fact, 85% of the population has been infected with adenovirus, and it is zero-positive for the presence of anti-adenoviral antibodies. Particularly, type 5 adenoviruses cause colds in children that, in most cases, are asymptomatic.

Different adenoviral vectors delectioned [literally translated "un-read" or "de-read"] in E1 have been used with little success for treating cancer in clinical trials. It has been proven that their efficiency was limited because of the small number of cells reached by the vector. The large size of the viral particle, 80 nm in diameter, makes its diffusion more difficult, and only a few tumor cell layers beyond the injection point or the blood vessels are reached by the vector. This limitation is particularly relevant in therapeutic approaches based on the introduction of cytotoxic or suppressor tumor genes, although a collateral cytotoxic effect has been observed in non-transduced cells near those that have been transduced. Even injecting multiple high doses of the vector, most of the tumor cells seem unaffected by the vector. In recent years, the selective spreading of the vector in tumor cells was used as a strategy for solving this limitation (R. Alemany et al., Nature Biotechnology 2000. Vol 18, pp. 723-7). Viral replication is, in and of itself, cytopathic, so cytotoxic genes or tumor suppressors are not necessary to achieve an anti-tumor effect. Somehow the concept of an adenovirus that is selectively replicated in tumor cells without having a non-viral gene, belongs more appropriately to the field of viral therapy or cancer virotherapy than to the gene therapy field. However, since cytotoxic genes, immunostimulators or tumor suppressors may stimulate the selective toxicity of the replicating adenovirus, such genes have been inserted into the

genome of the replicating adenovirus. Such selective replication vectors thereby meld virotherapy and gene therapy.

Virotherapy, or the use of viruses for treating cancer, predates gene therapy. The first records of curing tumors with viruses dates back to the beginning of the last century. There are viruses that display a natural oncotropism. For example, replication of the parvovirus seems to be related to the malignant transformation of the cell by an as yet unknown mechanism. The Vesicular Stomatitis Virus (VSV) displays an oncotropism that is associated with the observed antiviral effects of interferon. VSV is very sensitive to the interferon inhibition and often, tumor cells do not respond to the effects of interferon, so they display a deficient antiviral response. Another virus that was recently identified as oncotropic is the reovirus (Norman and Lee, Journal of Clinical Investigation. 2000. Vol 105, pp 1035-8). Infected cells react to the production of a double stranded RAN (dsRNA) during infection by the reovirus and other viruses by activating a kinase that depnds on the dsRNA (PKR). PKR, thus activated, blocks the synthesis of proteins by phosphorylating the alpha unit of the eIF2 translation factor. This blocking of the messenger RNA translation also blocks the viral RNA translation and thus, the replication of the virus. Many types of viruses express genes that inactivate the PKR but not the reovirus. However, PKR may be activated by other proteins located in the Ras signal translation path. Therefore, in cells with active Ras, as is the case with many tumor cells, reovirus may propagate. Other viruses do not display a natural oncotropism, but they may be genetically manipulated for selectively replicating themselves in tumors. For example, the Herpes Simplex Virus (HSV) has become oncotropic upon delection of the ribonucleotide reductase gene, a dispensable enzymatic activity in cells with active proliferation, such as tumor cells. Also, the HSV has become oncotropic by delecting the ICP34.5 protein counteracts the blocking of the PKR translation. Its delection results in an oncotropism by a mechanism similar to that of the reovirus. Recently, another virus that was manipulated to display an oncotropism is the influenza A virus (Bergman et al., Cancer Research 2001. Vol 61, pp. 8188-93). The NS1 viral protein of such virus also counteracts the blocking of PKR translation, and its delection results in a virus that is dependent on active Ras.

However, the adenovirus has been subject to more genetic manipulations to achieve selective replication in tumors. The starring role of the adenovirus in gene therapy for cancer, together with the experience amassed in clinical trials, has contributed to the popularity of such new replicating adenoviral vectors.

Two methods have been used for restricting the replication of adenovirus in tumor cells: the substitution of viral promoters by tumor selective promoters and the delection of viral functions that are not necessary in tumor cells. In both strategies, the gene to be regulated or mutated is preferably E1a, since it controls the expression of the other viral genes. Many tissue- or tumor-specific promoters have been used to control the expression of E1a. With regards to the strategy for delecting the viral functions that are not necessary for tumor cells, the first mutant proposed as selective replication, demonstrated an E1b-55K delection. That protein links and inactivates the p53 to induce the infected cell to enter the S phase S of the cellular cycle and to inhibit an apoptosis mediated by p53 that is be activated as a consequence of said induction. An E1b-55k mutated adenovirus, known as dl1520 or Onyx015, has been used for treating defective tumors in p53. Another mutation of the adenoviral genome to achieve a selective replication in tumors affects the CR1 and CR2 domains of E1a. Such domains of E1a mediate the union of proteins from the Retinoblastoma (RB) family. RB proteins block the transition of the G0/G1 phase to the S phase of the cellular cycle, forming an inhibiting complex of the transcription jointly with E2F. When E1a joins RB, the E2F transcription factor of the RB-E2F complex is liberated, and E2F acts as a transcriptional activator of the genes that are responsible for the transition to the S phase and of viral genes, such as E2. The liberation of E2F is thereby a key step for the replication of the adenovirus. In tumor cells, the cellular cycle is out of control since RB is absent or inactive for hyper-phosphorylation, and E2f is free. In said cells, the inactivating function of RB of E1a is no longer necessary. Therefore, the adenovirus with a mutation in E1a that prevents its union to RB may propagate normally in cells with inactive RB. The selective replication of such mutants has been already demonstrated (Fueyo et al., Oncogene 2000. Vol 19, pp. 2-12).

This invention describes a new type of mutation for achieving the selective replication in tumor cells with a determined genetic defect, other than using p53 and RB. Unlike other existing constructions in the field, this invention of DNA target of mutation does not produce a viral protein but a virus–associated RNA (VA), and it does not belong to early adenoviral genes but to the latent ones. Without any experimental data, WO 01/35970 mentions the use of a modified adenovirus wherein the VAI gene is not transcribed; however, in the technique, the joint use of mutated genes simultaneously in the VAI gene and the VAII gene has never been mentioned.

The genetic defect attacked in this invention is the transduction path of the Ras oncogene signal, a path that has not been previously attacked by the adenovirus. Many of the growth factors receptors activate Ras proteins (H-Ras, N-Ras, K-Ras and K-Ras B) to transmit a proliferating signal from the exterior of the cell to the nucleus. Ras proteins are little GTPases that, when joined a GTP, are able to activate a series of effectors. The activation of the effectors results in a mythogenic signal. Ras is mutated into a permanent active form in the 90% of pancreatic tumors, 50% of colon, 30% of lung, and in other proportions in many types of tumors. Apart from a large number of tumors with mutated Ras, the Ras path was found to be active in other cases by the constitutive activation of proteins that regulate Ras or the effectors of the Ras path. For example, the c-erbB gene that codifies the EGF receptor is frequently over-expressed in 50% of the glioblastomes and its homologue c-erbB2, is frequently over-expressed in breast and ovarian cancers. Generally, it is considered that the 80% of tumors have an activated Ras path. Many of said tumors, as in the case of pancreatic cancer, require new therapies due to the lack of response to the conventional therapy.

EXPLICATION OF THE INVENTION

The invention relates to the use of an adenovirus for cancer treatment, it is characterized by the fact that the adenovirus is defective in it associated RNAs (VA).

It also refers to the use of an adenovirus that has a mutation in the sequence of gene VAI or VAII.

Another purpose of this invention is the use of an adenovirus that has a mutation in the sequences that control the expression of the VA RNAs.

Another purpose of this invention is the use of an adenovirus that is injected into a tumor, into a cavity in which the tumor is located or into the blood stream of a cancer patient.

Another purpose of this invention is the use of an adenovirus, combined with other therapeutic modalities against cancer, such as chemotherapy and radiotherapy.

Another purpose of this invention is a composition comprised of an adenovirus with genetic mutations in the VA RNAs genes to achieve selective replication in tumor cells with an active Ras path or that are refractory to interferon action.

Even another purpose of this invention is a composition for use in cancer treatment, comprised of an adenovirus with genetic mutations in the VA RNAs genes and in one or more genes of the E1a, E1b and E4 group to achieve selective replication in tumors.

Even another purpose of this invention is a composition for use in cancer treatment that is comprised of an adenovirus with genetic mutations in the VA RNAs genes and of promoters regulating one or more genes of the E1a, E1b and E4 group to achieve selective replication in tumors.

Even another purpose of this invention is a composition comprised of an adenovirus with genetic mutations in VA RNAs genes to achieve selective replication in tumor cells and modifications in its capsid for increasing its infectiousness or to guide it to a receptor present in a tumor cell.

Even another purpose of this invention is a composition comprised of an adenovirus with genetic mutations in the VA RNAs genes resulting in selective replication in tumor cells and that contains other genes commonly used in cancer gene therapy field, such as pro-drugs activators, tumor suppressors or immunostimulators.

Even another purpose of this invention is a composition comprised of the human adenovirus derived from a serotype between 1 and 50, with genetic mutations in the VA RNAs genes resulting in selective replication in tumor cells.

This invention describes the use of mutant adenoviruses of the RNA VA genes for cancer treatment. RNA VA mutation allows the replication of the adenovirus to be conditioned on the existence of an active Ras path or on the inactivity of PKR due to a

lack of reaction to interferon. The invention addresses the necessity of finding better therapies for cancer of the pancreas, colon, lung and of other types of tumors.

This invention is comprised of an adenovirus that contains mutations in its genome that eliminate the function that inactivates the PKR of the virus-associated RNAs (VA). There are two genes codifying VA RNAs in the adenovirus genome, VAI and VAII, both located approximately at 30 units on the viral genome map. Both produce a short RNA (of about 160 ribonucleotides) that is synthesized by the RNA polymerase III in the later phase of the viral cycle. Each RNA VA is folded forming a curl that is joined with the kinase depending on the RNA, [i.e.,] the PKR. For purposes of propagating, the adenovirus uses the VA RNAs for inhibiting the PKR, otherwise said kinase phosphorylates the eIF2 proteins translation factor, inactivating it and thereby blocking the general synthesis of proteins. Therefore, the VA mutants described hereby are wrongly propagated in normal cells. On the contrary, in cells in which the PKR is already rendered inactive by the Ras path, as occurs in many tumor cells, said mutants are normally propagated. In cells that do not react to infection with adenovirus, inducing the PKR, VA mutants are also normally propagated.

The VA RNAs mutations of this invention may affect the VAI and VII genes. Whether alternatively or simultaneously, mutations may affect the promoters of the VAI or VAII genes or the transcription termination sequences that prevent their expression.

Adenovirus mutants in VA are propagated and amplified in cellular lines with the active Ras path and in the NP9 line of human pancreatic carcinoma. After their amplification in cell cultures, mutants are extracted and purified using standard methods in the adenovirology field.

Cancer is treated by the direct injection of the VA mutant into the tumor or by the systemic intravenous administration in cancer patients, using standard methods in adenovirus gene therapy.

DESCRIPTION OF DRAWINGS

The drawings included in the invention are attached for purposes of clarifying and providing a detail explanation of the characteristics, advantages and constructions of

the invention. Such drawings are part of the specifications and illustrate the preferred inventions but they must not be considered to limit the scope of the invention.

Figure 1: Secondary structure of the RNA VAI of serotype 5 adenovirus (Ad5).
The stem and handle structure is formed by matching the bases following the complementarity rules of Watson and Crick. The central domain is critical for the VA function, and the apical stem is also involved with the interaction of the RNA VAI with the PKR.

Figure 2: Sequence of the VA Ad5 region. The DNA sequence shown corresponds to base pairs 10500 to 11100 of the serotype 5 adenovirus genome. Such sequence goes from the base pair (bp) – 118, related to the beginning of the transcription of the VAI gene, to the 64 bp, beyond the termination point of VAII. The VAI gene (160 bp), from the beginning to the end of the transcription, is underlined and in italics. A 96 bp sequence separates the VAI and VAII codifying sequences. VAII (161 bp) is located after VAI and is underlined and in bold.

Figure 3: Selective Mechanism for the replication of defective adenoviruses in VA RNAs in cells with an active RAS path or that are refractory to interferon. Mechanism whereby virus associated (VA) RNAs mutants replicate, conditioned on the activation of Ras. Adenoviral infection produces double strand RNAs that induce the activation of PKR by phosphorylation. Activated PKR phosphorylates the translation factor of the eIF2 proteins and inactivates it, thereby blocking the general translation of proteins. VA RNAs of the adenovirus join and inactivate the PKR to counteract this antiviral response of the infected cell. Mutant adenoviruses of VA RNAs cannot inhibit the PKR and prevent the general blocking of protein synthesis. However, activation of the oncogenic path of Ras also inhibits the PKR, and when it is activated, VA mutants are propagate normally.

Figure 4: Affect of Ras activation on the propogation of VA RNAs. Graph of the production of the virus in 293 (replication, day 2). Cell line 293 shows low levels of

activated Ras. A plasmid containing an expression cassette of a negative dominant mutant Ras (RasN17) was transfected in 293, and the propagation efficiency of the mutant adenovirus RNA VAI (dl331) was evaluated. The Ras inhibition that can be observed in western blot can inhibit the dl331 propagation. On the contrary, when the 293 was transfected with a plasmid containing an expression cassette of a mutant that is constitutively Ras active (RasV12), Ras activation was observed was observed by the western blot, and dl331 propagation increased.

Figure 5: Propagation of a mutated adenovirus in RNA VAI in cells with low (293) or high (NPA) Ras activity. Graph of the cytopathic effect (CPE) quantified by BCA (day 5). Comparison of the VA RNAs mutant propagation in cells with low Ras activity and in pancreatic carcinoma cells with elevated levels of active Ras. Propagation of wild adenovirus Ad5 is used as a normalization control to correct the differences in the infectiousness and replication that may not be ascribed to VA mutation.

Figure 6: Treatment of tumors with RNA VA mutant. NP9 human pancreatic cancer tumors were implanted in immunosuppressed mice (naked mice Balb/c). When tumors reached a volume of 70-80 mm3, they were injected with RNA VAI (dl331) mutant adenovirus or with a control vehicle. Thereafter the tumor progression (tumor volume) was measured. The RNA VA mutant evidenced an anti-tumor effect.

DETAILED EXPLANTION OF THE PRODUCTION METHODS

Structure of mutated adenoviruses in virus associated (VA) RNAs.

This invention describes the use of mutated adenoviruses (i.e., functionally defective) in their genes codifying virus-associated (VA) RNAs for cancer treatment. Said treatment is based on the selective replication of VA mutants in cells with an active Ras path.

Moreover, tumors that are resistant to the antiviral effects of interferon (interferon alphas, betas and gammas) may also be treated with said mutants. The mechanisms that allow said replication conditioned on active Ras or on resistance to interferon, are detailed below.

In the cytoplasm of cells infected by adenoviruses, large quantities of some small RNAs, that are called associated to the virus or virus associated (VA), are detected. Said RNAs are synthesized by the cellular polymerase III RNA by the transcription of some adenoviral genes located approximately at 30 units on the adenoviral genome map. Some adenovirus serotypes just contain a VA gene (the ones that belong to subgroups A and F, and some serotypes that belong to group B), while others contain two VA genes (VAI and VAII that are present in some serotypes of subgroup B and in all serotypes of subgroups C, D and E). VA RNAs have about 160 ribonucleotides and are part of a secondary structure that is characterized by double stand stems and single strand curls (see Figure 1). This RNA VA competes with other double strand RNAs produced during the adenoviral infection to join a protein kinase called PKR. PKR is a kinase protein whose phosphorylation activity depends on the double strand RNA; however the union with RNA VA inhibits it, rather than activates it. This function of the VA RNAs is needed for viral replication since the activated PKR phospholylates the factor that starts the protein translation eIF2, by inactivating and blocking the protein synthesis. On the other hand, it has been disclosed that PKR may be inhibited by Ras (Mundschau and Faller, Journal Biological Chemistry, 1992. Vol 267, pp. 23092-8). With regards to PKR inhibition, Ras transduction path that is activated in a great number of tumors is functionally analogous to the VA RNAs. Connecting this observation, this invention establishes that in tumor cells with an active Ras path, VA RNAs functions may be eliminated without affecting viral replication. The invention, therefore, indicates that of VA RNAs mutants may be used for the treatment of tumors.

The selective replication mechanism in VA mutants tumors mentioned in the preceding paragraph is based on the fact that the effectors of Ras inactivate PKR. In many tumors, we also found another mechanism by which PKR is not activated: a lack of response to interferon. Interferon Secretion (IFN) of the alpha, beta or gamma types is the first response of the innate immune system against a virus. IFN induces the PKR expression, and the genes of the adenovirus VA RNAs antagonize the antiviral effects of IFN by inhibiting the PKR. In cells that do not respond to interferon, PKR is not induced, and the amount of PKR in the cytoplasm remains at very low base levels. In

that case, VA RNAs genes stop being necessary for allowing viral replication. It is well established that tumor cells display defects in their response to IFN. In fact, a virus that is very sensitive to inhibiting effects of IFN has been used for selectively lysing tumor cells and treating tumors (Stojdl et al. Nature Medicine 2000. Vol 6, pp 821-5). Connecting said observations, other configuration of this invention is the use of mutant adenoviruses of VA RNAs in the treatment of tumors with defects in the interferon path.

The gene sequence of serotype 5 adenovirus VA RNAs is shown in Figure 2. Gene VAI of adenovirus 5 is comprised of 160 pairs of bases, occupying base pairs 10620 to 10779 in the sequence of the adenoviral genome. Gene VAII is comprised of 161 base pair, occupying base pair 10876 to 11036. A configuration of this invention contains a delection within these sequences. Other configurations contain delections affecting sequences around them and that control the expression of VA genes. Particularly, sequences of 30 bases pairs before the VA genes have been described as being involved in the regulation of their expression. (Fowlkes and Shenk, Cell 1980. Vol 22, pp. 405-13). Another configuration contains delections of sequences after the VA genes that control the termination of their transcription by RNA polymerase III (Gunnery et al., Journal of Molecular Biology 1999. Vol 286, pp. 745-57).

During the study of the function of VA RNAs, several mutants were produced that eliminated their function. This invention establishes that the VA gene mutants that were previously produced and that eliminate their PKR inhibiting function may be used for cancer treatment. New VA RNAs mutants may also be used for the application described herein. There are several methods for manipulating the adenoviral genome. VA mutants may be produced, for example, by mutagenesis controlled by using the protocols previously published by the inventors, but instead of using adenoviral fragments of the hexon or the fiber described therein, using a fragment that contains VA genes. The procedure may be as follows: obtain purifed type 5 adenovirus DNA using K proteinase – SDS, using standard methods. Said viral DNA is cut with Kpn I restriction enzyme and a 2749 bp fragment (Ad5 bp # 8537 - 11286) that contains the VA RNAs genes and is purified by electrophoresis in gel. Said fragment is cloned by

ligation in a pUC19 plasmid with the same restriction enzyme. The mutagenesis directed for delecting any of the VA sequences mentioned above, is performed on said plasmid by using commercial protocols ("Quick Change site-directed mutagenesis kit", Stratagene, La Jolla, CA). The Kpn I mutated fragment is then introduced into the viral genome by a homologue recombination using a plasmid containing a complete genome of the Ad5, partially digested with Rsr II (the target in bp 109044 is repaired by the homologue recombination). From the resulting plasmid, we obtain the VA mutant by transfection of 293 cells or in cells with an active Ras path.

Other types of genetic mutations and manipulations different from the mutations of the VA RNAs genes described herein have been conducted to achieve selective replication in tumors. These may be insertions of active promoters in tumor cells for controlling the expression of viral genes and delections of early functions ("early E1 and E4") that block the RB or p53 paths. A configuration of this invention is the use of mutations in VA RNAs genes in combination with such other manipulations to achieve selective replication in tumors.

In another configuration of the invention, VA RNAs mutants may contain modifications in their capsids to increase their infectiousness or to be directed to receptors present in the tumor cell. Adenoviral capsid proteins were genetically modified to include ligands that increase infectiousness or direct the virus to a receptor in the tumor cell. The adenovirus to the tumor may also be achieved with bi-functional ligands that unite the virus, on one side, and the tumor receptor, on the other. In addition, to increase the persistence of the adenovirus in the blood and, thereby increase the possibilities of reaching the disseminated tumor nodules, the capsid may be covered by polymers, such as polyethylenglycol. These modifications may be configured in the VA RNAs mutants. Another configuration of this invention is the use of VA RNAs mutants of other adenovirus serotypes other than Ad5. Among the more than 50 human adenovirus serotypes, there are at least 47 serotypes in which the sequence of VA RNAs genes is well characterized (Ma and Mathews, Journal of Virology 1996. Vol 70,

pp. 5083-99). The mutation of VA genes in said serotypes may be used to achieve replication conditioned on active Ras or resistance to interferon.

Another configuration of this invention describes the use of mutant adenovirus VA RNAs that contain other genes for increasing their toxicity to tumor cells, such as the gene for the kinase thymidine, deaminase cytosine, proapoptotic genes, immunostimulators or tumor suppressors.

Production, purification and formulation of VA RNAs mutated adenovirus

Mutated adenovirus VA RNAs are propagated with the standard methods in adenovirology and adenoviral vector fields. The preferred propagation method is infection of a cell line that allows the replication of the mutant VA RNAs mutant. Said line has a mutated or active Ras oncogen for example. The NP9 pancreatic carcinoma line is an example of such line. For instance, propagation is performed as follows: NP9 cells grow on plastic cellular culture plaques and are infected using 50 viral particles per cell. Two days later, the cytopathic effect that reflects the production of the virus is observed as a bunching of cells. The cells are collected and stored in tubes. After a centrifugation at 1000g for 5 minutes, the cellular precipitate is frozen and unfrozen three times to break the cells. The resulting cellular extract is centrifuged at 1000g for 5 minutes, and the supernatant with viruses is carried [literally charged] on a cesium chloride gradient and centrifuged for 1 hour at 35,000g. The virus strip on the gradient is charge again on another cesium chloride gradient and is centrifuged for 16 hours at 35,000g. The virus strip is collected and is dialyzed with PBS-10% glycerol. The dialyzed virus is aliquoted and stored at -80°C. The number of particles and plaque-forming units forming are quantified using standard protocols. Phosphate-buffered saline solution with glycerol at 10% is a standard formulation for storing adenoviruses.

Use of mutant adenovirus of VA RNAs for cancer treatment

This invention describes the use of adenoviruses with defective VA RNAs genes for cancer treatment. The treatment is based on the selective replication of VA RNAs mutants in cells with an active Ras path or that are resistant to interferon.

Protocols for the use of VA mutants in cancer treatment follow the same protocols used by in virotherapy with adenovirus and in gene therapy with adenovirus. There is a wide experience in the use of non-replicating and replicating adenoviruses in the field of gene therapy. Particularly, adenoviruses with selective replication mechanisms, different than those proposed by this invention, have been used to treat cancer. There are many publications on the treatment of tumor cells in cultures, in animal models and in patient clinical trials. For the treatment of cells in *in vitro* cultures, the adenovirus, purified in accordance with any of the methods described above, is added to the culture medium to infect the tumor cells. For treating tumors in animal models or in human patients the adenovirus may be loco-regionally administered by injection in the tumor or in a body cavity where the tumor is located, or systemically by injection in the blood stream. As practiced with other selective replication adenoviruses, the treatment of tumor with VA RNAs mutants described as the purpose of this invention may be combined with other therapeutic modalities, such as chemotherapy or radiotherapy.

Example 1. Mutated adenovirus in the VAI gene displays replication dependent on Ras. To demonstrate that the replication of the RNA VAI (dl331) mutant is dependent on an active Ras path, we modulated the activation state of Ras in human cells. Approximately 1.0×10^7 human cells of the embryonic kidney (line 293) were seeded in a 10 cm diameter plaque and were transfected with 24 micrograms of plasmid containing either green fluorescence protein (GFP), the constitutively active form of Ras (H-Ras V12), or the dominant negative Ras (H-Ras N17). A standard protocol of calcium phosphate was used for transfection. Forty eight hours after the transfection, the cells were transferred to new plaques. To demonstrate the transfection effect of the plasmids in the Ras path, the levels of expression and of ERK phosphorylation (a Ras effector) in a cell lysed were measured using a Western-blot. Said lysing was obtained by incubation with subsidence buffer (20 mM Tris, 2 mM EDTA, 100 mM NaCl, 5 mM $MgCL_2$, 1% Triton X-100, 10% glycerol, 5 mM NaF, 100 microM Na_3VO_4, 1 mM PMSF, 10 micro/ml aprotinin, 10 micro/ml leupeptine) for 1 hour at 4°C. After centrifugation at 14,000xg, supernatant proteins (10 micrograms per rail determined by the Bradford

test) were electrophoretically separated in a gel of 10% polyacrylamide-SDS and were transferred to a PVDF membrane. The amount of ERK and phospho-ERK was measured by the Amersham chemi-luminescence protocol (ECL). A monoclonal antibody (Ab) was used a a primary antibody against the ERK (Zymed), [and] a polyclonal [antibody was used] against the phospho-ERK (Cell signaling Tech.) Mouse anti-IgG or rabbit anti-IgG, conjugated with radish peroxydase, was used as secondary antibodies. Following said proceedings we demonstrated that non-transfected cells 293 have a low ERK phosphorylation level, indicating a low activity of the Ras path. Controlled transfection with GPF does not affect said results. Transfection with H-Ras V12 increases ERK phospholylation, indicating the activation of the Ras path. On the contrary, transfection with H-Ras N17 results in the inhibition of the Ras way (Figure 4 of the invention, upper panel).

After verifying the modulation of the Ras path using the foregoing procedures, we begin to prove the selective replication of VA RNAs mutants, as described below. Transfected cells, as indicated in the preceding paragraph, were infected by RNA VAI dl331 mutant or with the wild-type adenovirus using 10 plaque-forming units per cell. Virus production was analyzed every day, measuring the quantity of adenovirus in the supernatant with 293 plaque formation trials. The wild-type adenovirus replicates 7 to 10 times better than the VAI mutant in 293 control cells or cells transfected with GFP. The activation of the Ras path induced by H-Ras V12 increased 10 times the efficiency of the VA mutant replication, so that its replication level reaches that of the wild-type adenovirus level. On the contrary, Ras path inhibition with H-Ras N17 decreased the replication of the VA mutant by two times. Therefore, compared to the wild-type adenovirus replication, RNA VAI mutant replication is 20 times more dependent on the activation of the Ras path that we have performed.

Example 2. Human tumor cells with active Ras paths allow the efficient replication of an adenovirus with a mutant RNA VAI.

Replication of a mutated adenovirus in an RNA VAI (dl331) gene was quantified in the NP-9 human pancreatic cancer line that contains a mutation in the codon 12 of the K-

Ras gene (GGT [becoming] GAT). Replication was estimated by the cytopathic effect (CPE) induced by the virus and measured as a decrease in the protein amount in the uni-layer cellular [sic] (BCA Method). In short, NP-9 cells were seeded in plaques of 96 cups with 30,0000 cells per cup. The following day, the cells were infected with serial dilutions of dl331 or of the wild-type adenovirus beginning with a concentration of 1000 plaque-forming units forming per cell. The infected cells were incubated for 5 days and the culture medium was removed to measure the amount of protein remaining in the cup. Figure 5 shows the results comparing the percentage of protein quantity vis-a-vis the cups that were not infected with the viral inoculums dilution. The dilution that produces 50% mortality (50% reduction of the protein content, IC_{50}) is an estimate of the oncolytic power of the initial preparation of the virus. In cells with mutated Ras (NP9), IC_{50} obtained for the RNA VAI dl331 mutant and for the wild-type adenovirus was 0.04 and 0.7 respectively, indicating an increase in potency of 18 times in the RNA VAI mutant (Figure 5, upper panel and continued line of the lower panel). In cells with low Ras activity (293), those values were 0.018 and 0.003 indicating a decrease in potency of 6 times in the RNA VAI mutant. As a whole, the results demonstrated that if we compare the oncolytic power of an RNA VAI mutant with the wild-type adenovirus in cells with active Ras, or in cells with almost inactive Ras, the Ras activation spurs the replication of the VAI mutant 100 times.

Example 3. An adenovirus mutated in the RNA VAI gene may be used for efficiently treating tumors.

Below, we demonstrate the anti-tumor effects of an RNA VAI (d1331) mutant adenovirus. An *in vivo* experiment was conducted with mice, athymic of the Balb/c strain containing tumors with an activated Ras path (NP9). All experiments were conducted pursuant to the guidelines of the FELASA ("Federation of European Labratory Animal Science Associations"). A total of 1.2×10^7 tumor cells of the NP-9 line cells were subcutaneously injected into each back flank of the mouse. After 15 days, the tumors forms (that reached 70-80 mm^3) were distributed in the different experimental groups (n=10 per group). Tumors of control group received two intratumor injections of saline buffer (2 x 10 microL). The tumors of the group treated

with the VA mutant received two intra-tumor injections (2 x 10 µl) of dl331 (10^9 viral particles per tumor). Figure 6 shows tumor volume at the beginning of the treatment (day 0). The results are shown as an average ± S.E.M. The existence of significant differences between the results was calculated using a non-parametric test of unmatched data of Mann-Whitney. Growth lines were compared using a variance analysis. The results were considered significant if $p<0.05$. The calculations were made with the SPSS statistic package (SPSS Inc., Chicago, IL). There is a significant difference between the tumor size on the 16^{th} and 21^{st} days. Tumors treated with mutant RNA VAI dl331 showed regression.

CLAIMS

1. The use of an adenovirus for the treatment of cancer characterized in that said adenovirus is defective in its virus-associated VA RNAsI and VAII.

2. The use according to claim 1, wherein said adenovirus has a mutation in the sequence of the RNAs VAI and VAII genes.

3. The use according to claim 1, wherein said adenovirus has a mutation in the sequences controlling the expression of the RNAs VAI and VAII genes.

4. The use according to any of the foregoing claims, wherein said adenovirus was injected into the tumor, into a cavity where the tumor is located, or into the blood stream of a cancer patient.

5. The use according to any of the foregoing claims, wherein said adenovirus is combined with other therapeutic modalities against cancer, such as chemotherapy or radiotherapy.

6. A composition comprised of an adenovirus with genetic mutations in VA RNAs genes to achieve selective replication in tumor cells with an active Ras path or that are refractory to the affects of interferon.

7. A composition to be used according to claims 1 to 3, which is comprised of an adenovirus with genetic mutations in VA RNAs genes, and in one or more genes of the E1a, E1b and E4 group to achieve selective replication in tumors.

8. A composition to be used according to claims 1 to 3, which is comprised of an adenovirus with genetic mutations in VA RNAs genes and also in promoters regulating one or more genes of the E1a, E1b and E4 group to achieve selective replication in tumors.

9. A composition comprised of an adenovirus with genetic mutations in VA RNAs genes to achieve selective replication in tumor cells and modifications in the capsid to increase its infectiousness or to direct it to a receptor present in a tumor cell.

10. A composition comprised of an adenovirus with genetic mutations in VA RNAs genes that results in selective replication in tumor cells and that also contains other genes that are commonly used in field of the cancer gene therapy, such as activators of pro-drugs, tumor suppressors or immunostimulators.

11. A composition comprised of a human adenovirus derived from a serotype between 1 to 50 with genetic mutations in VA RNAs genes that result in selective replication in tumor cells.